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## INCREASED DISSEMINATION OF DENGUE 2 VIRUS IN *Aedes Aegypti* ASSOCIATED WITH CONCURRENT INGESTION OF MICROFILARIAE OF *BRUGIA MALAYI*

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**Abstract.** We investigated whether concurrent ingestion of dengue 2 virus and microfilariae of *Brugia malayi* would increase viral infection and dissemination rates in *Aedes aegypti*. Infection rates were similar in mosquitoes that ingested virus alone or both virus and microfilariae concurrently. However, viral dissemination rates, as determined by recovery of dengue virus from both legs and bodies separately, were significantly greater in mosquitoes that ingested both agents concurrently than in those that ingested virus alone. This study confirms that vectorial capacity of a natural vector of an arbovirus may be enhanced by the concurrent ingestion of microfilariae.

Concurrent ingestion of microfilariae and virus by bloodfeeding arthropods may increase their vectorial capacity for the ingested virus.<sup>1,2</sup> This increased transmission may result from two separate effects. First, by perforating the midgut, microfilariae permit virus direct access to the hemocoel. This eliminates the need for a replication cycle in the midgut prior to viral dissemination to the hemocoel, thereby reducing the extrinsic incubation period. In addition, because mosquitoes are up to 1,000-fold more susceptible to viral infection by intrathoracic inoculation as compared to oral exposure,<sup>3,4</sup> the direct introduction of virus into the hemocoel would increase both infection, and more importantly, dissemination rates in potential vector species.

The effect of concurrent ingestion of microfilariae on the vectorial capacity of a mosquito for an arbovirus has been reported only for a *Brugia malayi*, Rift Valley fever (RVF) virus, and *Aedes taeniorhynchus* model.<sup>2</sup> In this system, mosquitoes that concurrently ingested microfilariae and RVF virus not only disseminated virus and became infectious more rapidly, but also trans-

mitted virus more efficiently than did a cohort that ingested similar quantities of virus from a gerbil infected with RVF virus alone. This model, however, is artificial. Rift Valley fever virus is a sub-Saharan African virus, *Ae. taeniorhynchus* is a North American mosquito, and *B. malayi* is a Southeast Asian filarial worm. Thus, it is necessary to confirm the phenomenon of increased vectorial capacity associated with concurrent ingestion of both microfilariae and virus by using a more natural system before arriving at a meaningful conclusion concerning the effect on viral transmission in nature.

We used an *Ae. aegypti*, dengue 2 (DEN-2) virus, and *B. malayi* model to investigate further the effect of concurrent ingestion of microfilariae on viral vectorial capacity. We viewed this model as more realistic because it incorporates components that occur together naturally in Thailand (i.e., *Ae. aegypti* is a vector of DEN-2 virus, local strains of both *Ae. aegypti* and DEN-2 virus were used in this experiment, and *B. malayi* occurs there). Also, the use of DEN-2 virus extends examination of this phenomenon to the Flaviviridae. We used the presence of virus in the mosquito legs (i.e., viral dissemination to the hemocoel) as our principal measure of vectorial capacity.

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## MATERIALS AND METHODS

*Mosquitoes*

Two strains of *Ae. aegypti* were used. One was collected as adults in Bangkok, Thailand, in January 1985 and provided by J. B. Gingrich of the Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand. This strain was designated as the THAI strain and was used in the F<sub>3</sub> generation. The other, BLACK EYE, is a long established African colony which has been maintained for an unknown number of generations at the Harvard School of Public Health.

*Viruses and viral assay procedures*

DEN-2 virus (025) from the blood of a dengue hemorrhagic fever patient in Bangkok, Thailand,<sup>5</sup> in 1978 was passed twice in LLC-MK<sub>2</sub> cells to provide a stock viral suspension. One ml, containing  $\approx 10^5$  plaque forming units (PFU) of this stock virus, was inoculated onto a monolayer of *Ae. albopictus* (C6/36) cells<sup>6</sup> in a T-75 tissue culture flask. After adsorption for 1 hr, 30 ml of L-15 medium with 10% heat-inactivated fetal bovine serum (FBS), 10% tryptose phosphate broth, and 50  $\mu$ g/ml gentamycin sulfate was added, and the cells were maintained in a humidified 5% CO<sub>2</sub> atmosphere for 6 days. The unfrozen supernatant from this flask was used as virus in this study.

Triturated mosquito specimens were assayed for dengue virus by plaque assay on BHK21-clone 15 cell monolayers.<sup>7</sup> A 0.1-ml aliquot of mosquito suspension (or serial 10-fold dilutions) was added to each of duplicate wells of 6-well (35 mm diameter) plastic tissue culture plates. After adsorption for 1 hr at 36.5°C, 3 ml of an overlay was added to each well. The overlay consisted of 0.75% agarose in Eagle's basal medium with Earle's salts with HEPES buffer (4 g/l), FBS (5%), and antibiotics (100 U penicillin, 100  $\mu$ g streptomycin, and 100 U mycostatin/ml). Six days later, a second overlay with neutral red (final concentration 1:5,000) but without FBS was added to each well to enhance visualization of plaques the following day.

*Microfilaremic gerbils*

A total of 6 male gerbils, *Meriones unguiculatus*, including 3 noninfected and 3 infected with

*B. malayi*, were used in this study. Approximately 8 months before their use in this study, gerbils were infected by subcutaneous inoculation of 200 third stage *B. malayi* larvae.

*Experimental design*

In order to produce a DEN-2 viremia, gerbils (both microfilaremic and amicrofilaremic) were anesthetized with sodium pentobarbital and inoculated with the never frozen C6/36 cell culture supernatant. About 0.3 ml of viral suspension was injected into the penile vein 10 min before mosquitoes were fed. The gerbils were placed separately on the screened surface of 3.8-l cardboard cages containing  $\approx 250$  female *Ae. aegypti* (THAI). After a 20 min feeding period, the gerbils were transferred to a second pair of cages containing  $\approx 250$  *Ae. aegypti* (BLACK EYE). At least 3 freshly engorged mosquitoes were removed from each cage to determine the amount of virus ingested. These mosquitoes were triturated individually in diluent (10% calf serum in Medium 199 with Hanks' salts plus 100 U penicillin, 100  $\mu$ g streptomycin, 5  $\mu$ g amphotericin B, 50  $\mu$ g gentamycin sulfate/ml, and 0.075% NaHCO<sub>3</sub>) and assayed for dengue virus as described above.

To determine the number of microfilariae ingested, at least 5 freshly engorged mosquitoes feeding on each microfilaremic gerbil were examined. Midguts were dissected and placed individually in wells of a 12-well (22 mm diameter) plastic tissue culture plate containing Lum's saline.<sup>8</sup> With a dissecting microscope at  $\times 45$  magnification, we determined the number of microfilariae that escaped the midgut after 1 hr. The number of microfilariae remaining in the midgut was determined by examining the squashed midgut under  $\times 100$  magnification with a binocular compound microscope.

The remaining engorged mosquitoes were transferred to 3.8-l cages. Cotton pledgets soaked in water and an apple slice were provided prior to placing the cages in an incubator maintained at 26( $\pm 1$ )°C with a 16L:8D photoperiod. A sample of 10–30 mosquitoes was removed from selected cages at intervals after the infectious bloodmeal. These mosquitoes were cold-anesthetized, their legs removed, and their legs and bodies triturated separately in 1 ml of diluent. Triturated specimens were frozen at  $-70^\circ\text{C}$  until assayed.

TABLE 1  
Viral infection rates in *Aedes aegypti* after ingestion of  $10^{1.5}$  PFU of dengue 2 virus from amicrofilaremic or microfilaremic gerbils

Days extrinsic incubation	Mosquito strains							
	THAI				BLACK EYE			
	Amicrofilaremic		Microfilaremic		Amicrofilaremic		Microfilaremic	
	% Inf.	No. exam.	% Inf.	No. exam.	% Inf.	No. exam.	% Inf.	No. exam.
6, 7	15	20	20	10	5	20	10	10
8, 9	25	20	15	20	10	20	5	20
10	26	91	23	60	18	80	13	40
Totals	24	131	21	90	16	120	10	70

## RESULTS

Mosquitoes that engorged on any 1 of 3 (2 amicrofilaremic and 1 microfilaremic) of the gerbils ingested  $\approx 10^{1.5}$  PFU of DEN-2 virus, while those feeding on a fourth gerbil (amicrofilaremic) ingested  $\approx 10^{1.2}$  PFU of virus. The 2 remaining gerbils (both microfilaremic) had viremias that were not detectable in a mosquito bloodmeal. Because we could not determine if these mosquitoes had actually been exposed to virus, they were discarded. This lack of viremia was probably due to our failure to introduce the DEN-2 viral suspension directly into the penile vein in these 2 gerbils.

Dissection of freshly engorged mosquitoes indicated that THAI and BLACK EYE strain mosquitoes, feeding on the dually-infected gerbil, ingested a mean of 7 and 8 microfilariae, respectively. Penetration rates were 54% and 72%, respectively, for these 2 strains. Even though *Ae. aegypti* failed to ingest detectable virus from the 2 remaining gerbils, they did ingest an average of 63 and 99 microfilariae from these gerbils. The percentage of ingested microfilariae that penetrated the midgut was relatively constant for mosquitoes that fed on the 3 microfilaremic gerbils, ranging from 50%–64% (mean 56%) for the THAI strain and from 39%–72% (mean 46%) for the BLACK EYE strain.

Virus was not detected in any of 71 mosquitoes tested on days 3 or 5 after the infectious bloodmeal, including 21 that had fed on the dually-infected gerbil. Beginning on day 6, however, virus was recovered from 23% of the THAI strain and 13% of the BLACK EYE strain that had fed on any of the gerbils with the higher viremias. Results from mosquitoes that ingested  $\approx 10^{1.5}$  PFU of DEN-2 virus from either of the 2 ami-

crofilaremic gerbils were combined and compared to those for mosquitoes that ingested a similar dose from the dually-infected gerbil.

For both strains of *Ae. aegypti*, there were no significant differences in viral infection rates between individuals that had ingested  $10^{1.5}$  PFU of DEN-2 virus alone and those that had ingested virus and microfilariae concurrently (Table 1). However, infection rates were significantly higher (*t*-test,  $P < 0.01$ ) in THAI (51/221, 23%) as compared to BLACK EYE (24/190, 13%) strain mosquitoes. Mosquitoes that ingested  $10^{1.2}$  PFU of dengue virus from an amicrofilaremic gerbil had a significantly lower (*t*-test,  $P < 0.001$ ) infection rate (3/110, 3%) than mosquitoes that ingested  $10^{1.5}$  PFU. Again, infection rates were higher for THAI (3/70, 4%) than for BLACK EYE strain (0/40) mosquitoes.

While infection rates were not affected by concurrent ingestion of microfilariae, dissemination of virus was enhanced by concurrent ingestion of microfilariae (Table 2). In the THAI strain, dissemination rates were 4 times higher (*t*-test,  $P < 0.01$ ) for mosquitoes that fed on the dually-infected gerbil (12%) than rates for those that fed on the amicrofilaremic gerbils (3%). Not only were dissemination rates higher, but viral dissemination also occurred more rapidly, day 6, as compared to day 10 for those mosquitoes that fed on the amicrofilaremic gerbil. Similarly, while no BLACK EYE mosquitoes (0/120) that fed on an amicrofilaremic gerbil 7–10 days earlier had a disseminated infection, 7% (5/70) of the mosquitoes that fed on the dually-infected gerbil had virus in their legs (*t*-test,  $P < 0.005$ ). Thus, in both mosquito strains tested, disseminated viral infections occurred earlier and at a higher rate in mosquitoes ingesting both agents as compared to those ingesting virus alone.

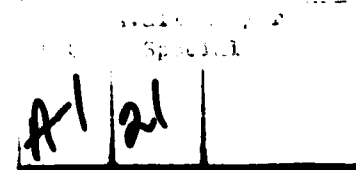


TABLE 2

Viral dissemination rates in *Aedes aegypti* after ingestion of  $10^{1.5}$  PFU of dengue 2 virus from amicrofilaremic or microfilaremic gerbils

Days extrinsic incubation	Mosquito strain							
	THAI				BLACK EYE			
	Amicrofilaremic		Microfilaremic		Amicrofilaremic		Microfilaremic	
	% Dis.	No. exam.	% Dis.	No. exam.	% Dis.	No. exam.	% Dis.	No. exam.
6, 7	0	20	10	10	0	20	10	10
8, 9	0	20	5	20	0	20	5	20
10	4	91	15	60	0	80	8	40
Totals	3	131	12	90	0	120	7	70

## DISCUSSION

Concurrent ingestion of microfilariae and DEN-2 virus significantly increased the potential for *Ae. aegypti* to transmit this virus. Thus, the present study supports similar findings for bluetongue virus and *Onchocerca cervicalis* in *Culicoides nubeculosus*<sup>1</sup> and for RVF virus and *B. malayi* in *Ae. taeniorhynchus*.<sup>2</sup> Also, the present study extends this phenomenon to include an additional family of viruses, the Flaviviridae. Enhanced arboviral transmission by arthropods, due to the concurrent ingestion of midgut-puncturing microfilariae, may be a general phenomenon which is an important factor in the epidemiology of many arboviral diseases.

Although we did not investigate transmission of DEN-2 virus in this study, numerous other studies have indicated that *Ae. aegypti* is a competent vector for all 4 dengue viruses. We found more rapid dissemination of dengue infection and an increased percentage of mosquitoes with a disseminated dengue infection in both strains of *Ae. aegypti* associated with concurrent ingestion of microfilariae. Thus, concurrent ingestion of microfilariae and DEN-2 virus should result in increased vectorial capacity for DEN-2 virus in these mosquitoes.

In vast regions of the tropics, long-lasting filarial infections are common. When arboviral infections occur in these areas, often the virus-infected vertebrate also will be microfilaremic. Thus, a significant proportion of arthropods feeding on a viremic host also might ingest microfilariae, and thereby greatly enhance their ability to transmit the arbovirus. Although filarial infections are often associated with the tropics, they are prevalent in the temperate regions as well. For example, in a 1961-1976 study, more than a quarter of white-tailed deer in the south-

eastern United States were infected with *Setaria yehi*,<sup>9</sup> and in a 1972-1973 study, at least half of the horses tested in the midwestern United States were infected with *O. cervicalis*,<sup>10</sup> the same filarial worm shown to enhance bluetongue viral infection in *C. nubeculosus*.<sup>1</sup>

Because microfilariae may allow for the introduction of virus directly into the hemocoel, their presence may enable an arthropod that is normally refractory to oral infection with a particular virus to develop a disseminated infection and subsequently transmit virus by bite. For example, Merrill and TenBroeck<sup>11</sup> found that *Ae. aegypti* that ingested eastern equine encephalitis (EEE) virus were unable to transmit that virus. However, when they punctured the midguts of these mosquitoes with a fine needle immediately after ingestion of EEE virus, these mosquitoes were able to transmit EEE virus by bite. Thus, if the hole punctured by the microfilariae is analogous to the needle stick, microfilariae could transform an incompetent vector into a competent one. The potential for viral infection in the hemocoel by virus escaping from the midgut is further enhanced by the relatively high susceptibility of mosquitoes to infection by intrathoracic inoculation as compared to their susceptibility following ingestion of virus.<sup>3,4,12</sup>

The mechanism by which the presence of microfilariae enables virus to enter the hemocoel directly has not been determined. It may be that a small portion of the viremic bloodmeal escapes into the hemocoel through the holes punctured in the midgut by the penetrating microfilariae. Alternatively, virus may adsorb to the sheath of the microfilariae and be carried actively through the midgut wall into the hemocoel. This adsorption could occur anytime up to, or even shortly after, the bloodmeal is ingested by the arthropod. Most *B. malayi* microfilariae appear to complete

exsheathment shortly after they penetrate the midgut wall.<sup>13</sup> Thus, virus adsorbed to the sheath might become disassociated in the hemocoel. This latter mechanism may help explain the 24- to 48-hr delay in the detection of disseminated infections with RVF virus in *Ae. taeniorhynchus* after ingestion of this virus and microfilariae of *B. malayi* as compared to individuals inoculated intrathoracically with this virus (M. J. Turell, personal communication). Two possible explanations for our failure to detect DEN-2 virus until 6 days after the infectious bloodmeal could be an increased time for disassociation of DEN-2 virus from the sheath or the slower rate of replication of this virus in mosquitoes.

In our study, we produced an artificial viremia only in the gerbil with the lowest microfilaremia. As mosquitoes ingested (and were penetrated by) nearly 10 times as many microfilariae from the 2 gerbils in which we failed to create a detectable viremia, it is possible that the effect of concurrent ingestion of DEN-2 virus and microfilariae would have been greater in mosquitoes feeding on these 2 gerbils.

Ingestion of microfilariae along with an arbovirus appears to enable that virus to bypass both the midgut infection and dissemination to the hemocoel phases of the replication cycle in the potential vector. This not only increases the number of individuals capable of transmitting the virus, but also reduces the extrinsic incubation period. Because of the relatively high prevalence rates and long persistence of filarial infections in many parts of the world, the increased vectorial capacity associated with dual infections should be considered in control programs aimed at either the arbovirus or the filarial worm.

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